EFFECTS OF BOVINE POLYMERIZED HEMOGLOBIN ON COAGULATION IN CONTROLLED HEMORRHAGIC SHOCK IN SWINE

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ABSTRACT—HBOC-201, a bovine polymerized hemoglobin, has been proposed as a novel oxygen-carrying resuscitative fluid for patients with hemorrhagic shock (HS). Herein, we evaluated the hemostatic effects of HBOC-201 in an animal model of HS. A 40% blood loss-controlled hemorrhage and soft tissue injury were performed in 24 invasively monitored Yucatan mini-pigs. Pigs were resuscitated with HBOC-201 (HBOC) or hydroxyethyl starch (HEX), or were not resuscitated (NON) based on cardiac parameters during a 4-h prehospital phase. Afterward, animals received simulated hospital care for 3 days with blood or saline transfusions. Hemostasis measurements included *in vivo* bleeding time (BT), thromboelastography (TEG), *in vitro* bleeding time (platelet function; PFA-CT), prothrombin time (PT), and partial thromboplastin time (PTT). Serum lactate was measured and lung sections were evaluated for microthrombi by electron microscopy. During the prehospital phase, BT remained unchanged in the HBOC group. TEG reaction time increased in HBOC pigs during the late prehospital phase and was greater than in NON or HEX pigs at 24 h ($P = 0.03$). TEG maximum amplitude was similar for the two fluid-resuscitated groups. PFA-CT increased in both resuscitated groups but less with HBOC ($P = 0.02$) in the prehospital phase; this effect was reversed by 24 h ($P = 0.02$). In the hospital phase, PT decreased ($P < 0.02$), whereas PTT increased above baseline ($P < 0.01$). Lactic acidosis in HBOC and HEX groups was similar. Aspartate aminotransferase was relatively elevated in the HBOC group at 24 h. Electron microscopy showed no evidence of platelet/fibrin clots or microthrombi in any of the animals. Twenty-four-hour group differences mainly reflected the fact that all HEX animals (8/8) received blood transfusions compared with only one HBOC animal (1/8). In swine with HS, HBOC resuscitation induced less thrombopathy than HEX during the prehospital phase. Mild delayed effects on platelet and clot formation during the hospital phase are transient and likely related to fewer blood transfusions. In swine with HS, HBOC resuscitation induced less thrombopathy than HEX during the prehospital phase but more thrombopathy in the hospital phase. The delayed effects on platelet and clot formation during the hospital phase are transient and may be related to the need for fewer blood transfusions.

KEYWORDS—Blood substitutes, hemoglobin-based oxygen carriers, trauma, coagulopathy, resuscitation

INTRODUCTION

Severe hemorrhage accounts for about 30% of civilian trauma and 50% of combat deaths (1, 2). Approximately 60% of these casualties are potentially salvageable if blood loss is under 50% of estimated blood volume (2). Recovery and management of such hemorrhagic shock (HS) casualties is critically dependent on the response time to administration of advanced life-support procedures. Resuscitation in HS targets restoration of intravascular volume, tissue oxygenation, and hemostasis to prevent complications such as vascular collapse, tissue hypoxia, lactic acidosis, and coagulopathy (3). Approximately 39% of HS patients that survive 24 h posthospital admission develop multiple organ system failure (4, 5). Colloid-based resuscitation fluids such as buffered hydroxyethyl starch restore blood pressure but have limitations with respect to balancing other homeostatic parameters such as

coagulation and tissue oxygenation. Such limitations may contribute to the subsequent development of multiple organ system failure. Hemoglobin-based oxygen carrier (HBOC) fluids have been proposed as more comprehensive resuscitation fluids (6), and have been shown to substitute for blood transfusion in surgical clinical trials (7) and to increase survival in animal models of controlled and uncontrolled hemorrhage (8–10).

Coagulopathy/thrombopathy affects approximately 30% of patients with severe trauma, and is often defined as a 1.5- to 2-fold increase in prothrombin time (PT) and partial thromboplastin time (PTT) (11). These effects are believed to involve at least two mechanisms: coagulation factor consumption (after onset of stress) and dilution by resuscitation fluids. Active components of the fluid can also affect hemostasis parameters (11, 12). Platelet activation is enhanced when endothelium is disrupted, collagen is exposed, and tissue factor is released. In addition, excessive fibrinolysis leading to disseminated intravascular coagulation (DIC) also occurs in HS and has been correlated with a high Injury Severity Score (13). Hepatic function is also challenged in response to HS; the hypermetabolic component of the initial stress response to HS may interrupt hepatic synthetic function and depress production of proteins

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and enzymes. The inability to produce albumin, for example, may alter colloid properties of blood (11).

Large volumes of crystalloid fluids are required for the restoration of blood pressure, whereas HBOC-201 has been proposed as a low-volume resuscitative fluid (9). Colloids such as hydroxyethyl starch are known to affect coagulation and platelet function based on polymer molecular weight (14). Starches may cause hypocoagulation by impairing von Willebrand factor (vWF) function as in vWF disorder (15). The effects of HBOC on coagulation and hemostasis have not been extensively studied. Potential HBOC resuscitation-related hemostatic effects include hemodilution, decreased cellular mass and nitric oxide (NO) scavenging, and possibly platelet activation (16). Herein, we report effects of HBOC-201 and Hextend (HEX), the resuscitation fluid of choice used by the military, on hemostasis and thrombosis in a swine model of HS induced by controlled hemorrhage and associated soft tissue injury.

MATERIALS AND METHODS

These experiments were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996). The study was approved by the Walter Reed Army Institute of Research/Naval Medical Research Center Institutional Animal Care and Use Committee. All procedures were performed in an animal facility approved by the American Association for Accreditation for Laboratory Animal Care.

Animal and hemorrhage model

The study was divided into a 4-h prehospital phase simulating an "evacuation delay" period during which blood transfusions and surgical stabilization were unavailable, as is common in military operations. This was followed by a 3-day hospital phase where surgical repair of open-catheter technique and laparotomy wounds, and blood transfusions were available. Swine were used in a model simulating traumatic HS in the battlefield caused by a vascular injury in which hemorrhage was controlled. Specifically, 24 Yucatan mini-pigs (26.8 ± 4.3 kg) were anesthetized (ketamine/isofluorane induction and isofluorane maintenance), intubated, and allowed to breath spontaneously (FiO$_2$ = 0.21). Rectal temperature was monitored and body heat was maintained. The external jugular vein and carotid artery were catheterized by open technique for vascular access and continuous blood pressure monitoring. A pulmonary artery catheter was also inserted. To mimic soft tissue injury, the rectus abdominus muscle was crushed in a standardized fashion for 5 min with a Kocher clamp. After an equilibration period (5–10 min), animals were hemorrhaged to 40% estimated blood volume by catheter withdrawal of blood over 15 min (~1.7 mL/kg/min).

Time 0 designated initiation of the rectus abdominus crush and concomitant hemorrhage. To simulate battlefield delay in initiation of resuscitative measures, animals were left in shock for an additional 5 min (time 20 min). Animals were then infused resuscitation fluids at 10 mL/kg over 10 min. Subsequently, animals were fluid resuscitated with 5 mL/kg over 10 min at 30, 60, 120, and 180 min, if prospectively defined criteria were met (i.e., mean arterial pressure [MAP] < 60 mmHg or heart rate > baseline). Animals were intensively monitored in this prehospital phase (total of 4 h), but received only fluid resuscitation. Afterward, hospital care was simulated by surgical repair and the availability of blood transfusions. Carotid and pulmonary arterial catheters were removed, and neck and abdominal skin and fascia was closed. At 4, 24, and 48 h, animals received matched whole blood transfusions (for hemoglobin [Hb] < 7 g/dL) or normal saline (for Hb > 7 g/dL) at 10 mL/kg. Shed blood was collected in blood bags containing standard anticoagulant (CPD-A; Fenwal, IL) and stored for potential use in autologous or allogenic transfusions during the hospital phase (all pigs were blood type A). Animals were euthanized 3 days postoperatively. Vital signs and physiologic monitoring were performed as described by Philbin et al. (10). Most physiology data are briefly summarized in the "Results."

Fluid resuscitation

Swine were randomly allocated to one of three resuscitation study groups: HBOC-201 (HBOC; n = 8), HEX (n = 8), and nonresuscitated (NON, n = 8). HBOC is purified and ultrafiltered bovine, stroma-free Hb, heat-treated and polymerized by gluteraldehyde-crosslinking into polymers ranging from 130 to 500 kD. HBOC is prepared in a buffer similar to lactated Ringer's containing a 50:50 racemic D- and L-lactate mixture with *N*-acetyl-poly-cysteine. HBOC contains approximately

13 g Hb/dL, and has an oncotic pressure of 17 mmHg, an osmolality of 300 mOsmol/kg, and an oxygen affinity (P$_{50}$) of 38 mmHg, which is lower than human blood. HBOC-201 is stable at 25°C for at least 3 years (17). HEX is 6% hydroxy-ethyl starch (with a molecular weight of 670 kD) prepared in balanced lactated Ringer's (50:50 racemic mixture; Hextend, Abbott Laboratories, Abbott Park, IL).

Bleeding time (BT)

BT was measured at time 0 and 4 h posthemorrhage (at 4 h, the MAP had stabilized). BT was performed by incision with a scalpel blade (no. 11) on an ear edge to create a reproducible 5-mm anterior incision. The time for the bleeding to stop was recorded using the paper blotting method using Whatman paper no. 1 (Clifton, NJ).

In vitro *monitoring*

Blood sample collection—Blood was collected in vacutainer tubes (BD Vacuutainer, Palo Alto, CA) without anticoagulant (chemistry assays), with 3.2% citrate (coagulation assays), or sodium heparin (lactate assay). Blood samples were collected at time 0, 30, and 60 min, and 3 and 4 h before scheduled fluid infusions. After animals recovered from anesthesia, blood was collected at 24, 48, and 72 h. Phlebotomy blood volumes were not included in reported hemorrhage volumes.

Hematology

Complete blood count with differential was measured on a Pentra 60C+ cell counter (ABX Diagnostics, Irvine, CA). Plasma hemoglobin (due to HBOC) was detected with the B-hemoglobin method (Hemocue, Angelholm, Sweden) (18).

Assays

All functional laboratory assays were performed at 37°C, consistent with animal rectal temperatures (36.9°C ± 1.3°C). Laboratory studies included PT, PTT, thrombin time, antithrombin (AT-III), fibrinogen, thromboelastography (TEG).

TEG

TEG was performed to study clot formation dynamics (Haemostasis Analyzer; Haemoscope Corp., Niles, IL). Twenty microliters of 0.25 mM CaCl$_2$ and 340 μL of whole blood was pipetted into an oscillating cup with a 45° angle motion. A pin inserted into the cup is connected to a torsion wire that monitors cup torque during clot formation. The analog signal was computerized to give parameters such as: reaction time (TEG-R), which corresponds with initiation of fibrin formation and depends mainly on plasma factors; kinetics of clot formation, TEG-K and TEG-α, as measurements of platelet adhesion on newly formed fibrin and rate of fibrin polymerization, respectively; and TEG-MA, which depends on platelet number and function, and to a lesser extent on plasma proteins (19). TEG-Ly measures fibrinolysis (at 30 min), mainly due to tissue plasminogen activator, and is indicative of the presence of the fibrin(ogen) degradation product. The coagulation index (TEG-CI) is a computed index (19): TEG-CI = (0.0184 × TEG-K) + (0.1655 × TEG-MA) − (0.0241 × TEG-α) − (0.2454 × TEG-R) − 5.022. Standards and controls were run to assure optimal instrument performance.

PFA-CT

The platelet function analyzer (PFA-100; Dade Behring, Deerfield, IL) measures closure time in an ADP collagen capillary and corresponds with *in vitro* BT (20). Whole blood (800 μL) was vacuum aspirated through a 100-μm diameter capillary. The capillary membrane was coated with collagen and ADP, which promote platelet adhesion/aggregation, platelet plug formation, and arrest the flow of blood. Time to aperture occlusion is referred to as the CT, which is increased by low hematocrit, low platelet count, qualitative platelet defects, and qualitative/quantitative vWF deficiencies; CT is unaffected by coagulation factor deficiencies and hypofibrinogenemia (20).

Coagulation assays

Coagulation assays were performed on a Stat Compact (Diagnostica Stago, Parsippany, NJ) according to the manufacturer's instructions using Diagnostica Stago reagents. This is a fully automated instrument using electromagnetic principles for PT, PTT, thrombin time, and fibrinogen, and colorimetric principles for AT-III. (Note: interference by HBOC in plasma precluded colorimetric measurement of AT-III).

Chemistry

pH was measured in citrated blood at 24°C with a pH meter (WTW, Welheim, Germany), which was calibrated daily. Total protein, albumin, lactate, and aspartate aminotransferase (AST) were measured in heparinized blood on a Vitros 250 (dry chemistry; Ortho-Clinical Diagnostics, Raritan, NJ). For lactate, no interference was seen with HBOC concentration up to 6.5 g/dL. Blood samples were diluted 3-fold for AST assays due to AST levels above the upper limit of detection of the Vitros 250 instrument, which brought the level of HBOC below the 2.5 g/dL interference limit.

Detection of microthrombi and fibrin deposition

Electron microscopy (EM) was performed on lungs after necropsy as previously described (21). Briefly, lungs were fixed in 4F1G fixative (4% paraformaldehyde and 1% gluteraldehyde) overnight, postfixed in 2% osmium tetroxide, dehydrated in graded alcohols, and embedded in epon 812. Block sections (1-μm thickness) were examined by light microscopy, and thin (90-nm) sections were stained with lead citrate and uranyl acetate, and were examined with a LEO 912 AB electron microscope.

Statistics

Results, data, and figures are presented as means ± SD unless otherwise stated. Data were analyzed using the mixed model. Significant group and/or time effects were subsequently compared using a two-tailed paired Student's t test assuming equal variance. $P \le 0.05$ was considered statistically significant.

RESULTS

In vivo monitoring (10)

Baseline MAP was comparable in all three groups (HBOC, 71.1 ± 8.0 mmHg; HEX, 62.0 ± 9.8 mmHg; and NON, 61.0 ± 10.5 mmHg).[10] Posthemorrhage (15 min), MAP dropped significantly in all groups (26.3 ± 7.9 mmHg). HBOC restoration had a more pronounced effect on blood pressure restoration (at 30 min, MAP was 63.4 ± 20.9 [HBOC] compared with 37.3 ± 12.7 mmHg [HEX] and 35.4 ± 19.7 mmHg [NON], P = 0.01). After fluid resuscitation, MAP surpassed baseline throughout most of the prehospital phase in the HBOC group, probably mainly because of the NO scavenging properties of HBOC (16, 17). For example, compared with baseline, at 4 h, MAP was higher in HBOC pigs (92.3 ± 20.6 mmHg, $P < 0.02$), comparable in HEX pigs (61.8 ± 15.5 mmHg, $P > 0.05$), and lower in NON pigs (54.0 ± 19.2 mmHg, $P < 0.02$). Total prehospital fluid requirements were less with HBOC pigs (515 ± 179 mL or 18.8 ± 1.8 mL/kg) than with HEX pigs (847 ± 137 mL or 29.9 ± 1.1 mL/kg; $P < 0.01$). The mean hemoglobin load in HBOC infusions was 2.35 g/kg, resulting in a mean peak plasma concentration of 5.0 ± 1.2 g/dL at 4 h, as well as residual 2.6 ± 1.0 g/dL at 24 h. The half-life of HBOC was ~22 h. Cutaneous tissue oxygenation was significantly improved with HBOC ($P < 0.001$; e.g., at 60 min, 26.2 ± 6.6 vs. 9.8 ± 3.6 mmHg in HBOC and HEX pigs, respectively). Seventy-two hour survival was 100% (8/8) with HBOC pigs, 88% (7/8) with HEX pigs, and 63% (5/8) in NON pigs ($P > 0.05$). The four early deaths occurred between 90 and 800 min posthemorrhage.

Hospital phase fluid requirements

From 4 to 48 h, blood or saline transfusions were available to the animals. Fewer blood transfusion were required in HBOC than HEX pigs ($P < 0.01$; e.g., at 4 h, 0/8 [HBOC] vs. 6/8 [HEX] received 0 vs. 208 ± 138 mL blood, respectively). The NON group required more saline infusions than the treated animals (e.g., at 4 h, 0/8 vs. 7/7 in HBOC and NON, respectively; Table 1).

In vivo BT

In NON-resuscitated pigs, BT shortened from 112 (time 0) to 70 s (time 4 h; $P < 0.05$); in contrast, there were no significant changes in BT in the fluid-resuscitated groups ($P > 0.05$) between 0 and 4 h. Also, there were no detectable differences in BT between HEX and HBOC groups at 4 h ($P > 0.05$; Fig. 1).

In vitro results

Hematology—Hematocrit (Hct; baseline, 29.36% ± 2.58%) decreased to ~15% at 4 h in the HBOC and HEX groups (likely

TABLE 1. **Hospital phase fluid requirements**

	4 h	24 h	48 h	Total
Blood volume transfused (mL)				
HBOC	0*	22 ± 63	0	22 ± 63
HEX	208 ± 132	31 ± 81	23 ± 60	111 ± 256
NON	0	101 ± 143	40 ± 89	101 ± 192
Saline volume infused (mL)				
HBOC	0#	113 ± 155	126 ± 141	229 ± 289
HEX	63 ± 119	90 ± 154	0	142 ± 155
NON	310 ± 78	54 ± 122	40 ± 89	378 ± 211

Mean (±SD) blood volume (mL) transfused and saline volume infused per surviving animal at 4, 24, and 48 h, and total volume.
*$P < 0.01$ for blood transfusion comparisons in HBOC and HEX animals at hospital arrival. #$P < 0.01$ for saline infusion comparison in HBOC- and NON-animals at hospital arrival.
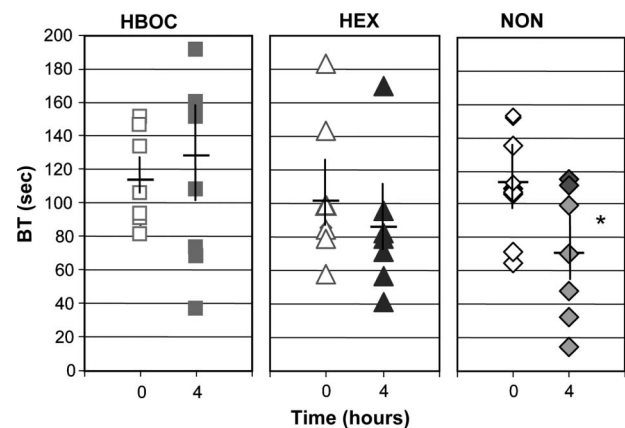

FIG. 1. **BT in 40% controlled hemorrhaged swine.** *In vivo* BT at time 0 (open symbol) and 4 h (closed symbol) in HBOC (■), HEX (▲), and NON (♦) pigs for surviving animals. Values are presented as individual data, and bars indicate mean ± SD. *$P < 0.05$.

hemodilution-related; Fig. 2A). During the hospital phase, Hct increased in both groups because of blood transfusions (and possibly erythropoiesis). In contrast, Hct increased in NON pigs during the prehospital phase and decreased after 4 h because of hemodilution by blood or saline. Hemoglobin (baseline, 10.32 ± 0.93 g/dL) paralleled the Hct in HEX and NON pigs but stabilized in HBOC pigs due to hemoglobin delivery by HBOC (Fig. 2B). There was a greater decrease in platelet concentration (baseline, $417 ± 63 \times 10^6$/mL) in the two treatment groups (group effects, $P < 0.01$; Fig. 2C). The platelet concentration rebounded in the hospital phase most rapidly in HEX pigs, intermediate in HBOC pigs, and slowest in NON pigs (Fig. 2C). The total platelet number during the prehospital phase was normalized to the hematocrit. This calculation indicated that the total number of circulating platelets was comparable in all three groups (Fig. 2D).

The white blood cell concentration (baseline, $19.4 ± 3.8 \times 10^6$/mL) increased in the NON group and decreased in both treated groups. In all three groups, monocytes increased at 24 h and remained elevated throughout the hospital phase. All three groups exhibited similar changes in the percentage of neutrophils (peaking at 4 h) and lymphocytes (decreasing at this time; results not shown).

There were significant differences between the TEG-R curves (group effect, $P = 0.004$). TEG-R (baseline, 4.88 ± 1.88 min) was unchanged during the prehospital phase for NON and HEX animals. In HBOC animals, TEG-R was significantly greater than HEX and NON animals at 24 h ($P < 0.05$; Fig. 3A). TEG-K (baseline, 1.24 ± 0.46 min) showed the same pattern as TEG-R. TEG-α (baseline, 73.61 ± 4.21 degrees) expressed a mirror image of this pattern (data not shown). TEG-MA (baseline, 72.36 ± 5.86 min) was unchanged in NON animals but decreased $\sim 25\%$ in HBOC and HEX animals during the

prehospital phase (Fig. 3B). These values returned to baseline in the hospital phase similarly in both treatment groups. TEG-CI (Fig. 3C) supported the other TEG findings. TEG-CI was lower in the resuscitated than the NON groups by 3 and 4 h ($P < 0.05$). During the hospital phase, TEG-CI returned to normal in the HEX group at 24 h and the HBOC group at 48 h. TEG-Ly (baseline, $2.95\% \pm 1.85\%$) varied significantly between groups (group effect, $P < 0.001$; Fig. 3D). All values remained near baseline during the prehospital phase and decreased at the onset of the hospital phase.
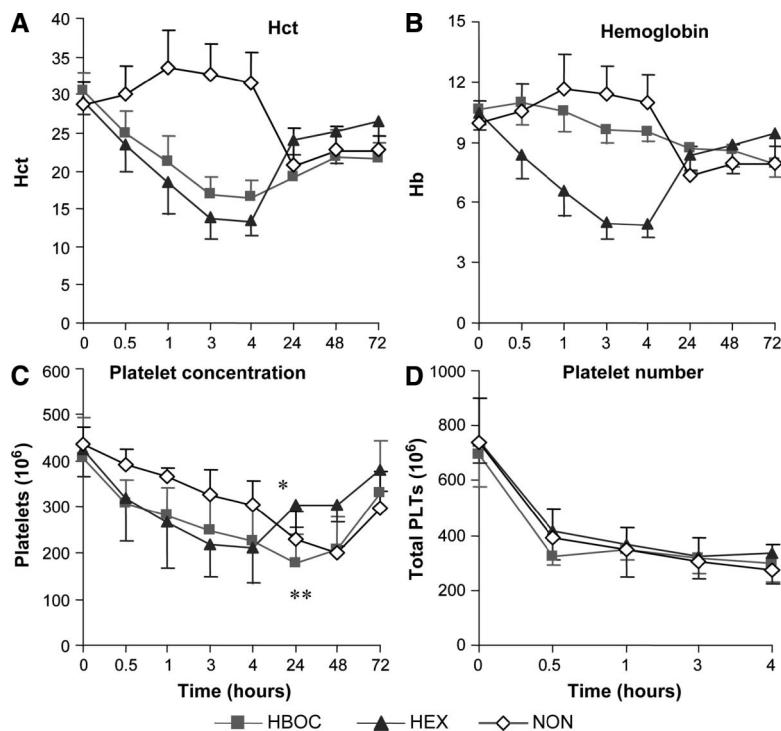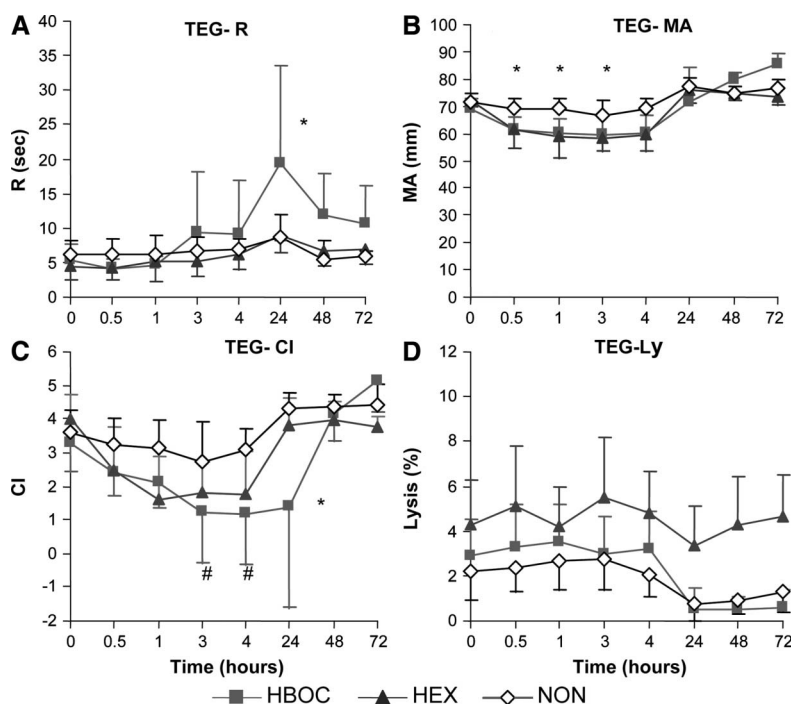

Fig. 2. **Hematology parameters for 40% controlled hemorrhaged swine**. Data for HBOC- (—■—), HEX- (—▲—), and NON-resuscitated pigs (—◇—). (A) Hct, (B) Hemoglobin, (C) platelet concentration, and (D) total platelets (calculated from the platelet concentration × volume of blood and fluid *in vivo*). Values are presented as mean ± SD. *$P < 0.02$ for HEX between 4 and 24 h. **$P < 0.001$ between HBOC versus HEX at 24 h.

Fig. 3. **TEG for 40% controlled hemorrhaged swine**. TEG for HBOC- (—■—), HEX- (—▲—), and NON-resuscitated pigs (—◇—). (A) TEG-R; (B) TEG-MA; (C) TEG-CI; and (D) TEG-Ly. Values are presented as mean ± SD. *$P < 0.05$ between HBOC and NON animals. #$P < 0.05$ HBOC versus NON and HEX versus NON (3 and 4 h).

The *in vitro* PFA-CT (baseline, 58.85 ± 13.05 s) curves were significantly different (group effect, $P < 0.001$). During the prehospital phase, PFA-CT increased only in the resuscitated groups. At 3 h, PFA-CT was higher in HEX than HBOC animals ($P < 0.02$). After 24 h, PFA-CT decreased in HEX animals but continued to increase and remained elevated at 24 and 48 h in HBOC animals ($P < 0.05$). In all cases, PFA-CT values returned to baseline by 72 h ($P = 0.05$; Fig. 4).

Coagulation results

Coagulation parameters vary with temperature and pH. The temperature in the studied pigs was controlled at 36.9°C ± 1.3°C (average) and ranged from 34.1°C to 41.7°C. pH of citrated blood samples (baseline, 7.40 ± 0.06) remained fairly constant for HBOC and NON animals during the 4-h prehospital period (7.40 ± 0.04 and 7.36 ± 0.04, respectively) but dropped significantly at 3 and 4 h in HEX animals ($P < 0.05$; Fig. 5A). Lactate (baseline, 1.8 ± 0.8 mM) was not significantly different across treatment groups. The peak at 3 h in the NON group was caused by high lactate in nonsurviving animals (5.6 ± 2.3 nM).

PT (baseline, 14.0 ± 2.2 s) was unchanged throughout the prehospital phase in NON animals, however, during the hospital phase (after 48 h), PT was lower ($P = 0.02$). Although PT gradually increased in HEX animals during the prehospital phase, this did not reach statistical significance. During the hospital phase, PT was similar across all three treatment groups (Fig. 6A).

Although there were no significant group differences, PTT and fibrinogen varied over time (time effect, $P < 0.001$). In the NON group (baseline, 24.7 ± 3.8 s), PTT was constant during the prehospital and hospital phases. However, PTT decreased by 25% in the resuscitated groups early in the prehospital period and increased above baseline in the hospital phase ($P < 0.01$; Fig. 6B). Fibrinogen (baseline, 168 ± 38 mg/dL) decreased in the treated animals in the prehospital phase and increased in all groups in the hospital phase (Fig. 6C). AT-III (baseline, 96% ± 10%) slowly increased in the NON group up to 72 h (111% ± 10%); at 4 h, AT-III was reduced because of
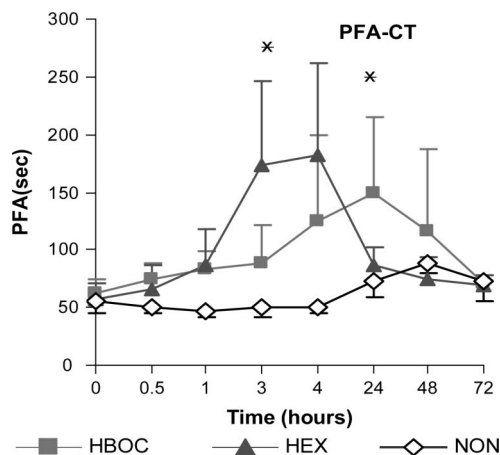
Fig. 4. ***Ex vivo* BT (PFA-CT) in 40% controlled hemorrhaged swine.** PFA-CT, for HBOC- (—■—), HEX- (—▲—), and NON-resuscitated pigs (—◇—). Values are presented as mean ± SD, *$P < 0.05$ between HBOC and HEX.
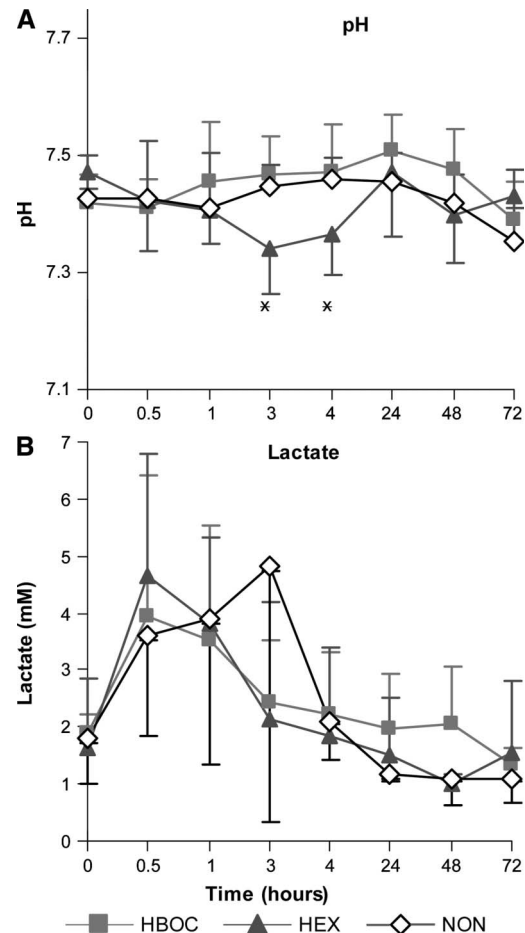
Fig. 5. **pH and lactate measured in 40% controlled hemorrhaged swine blood samples**. (A) pH measured on citrated samples and (B) L-Lactate after treatment with HBOC- (—■—), HEX- (—▲—), and NON- resuscitated pigs (—◇—). Values are presented as mean ± SD, *$P < 0.05$ from NON.

hemodilution (55% ± 9%) in HEX animals and returned to baseline by 24 h (113% ± 4% at 72 h; data not shown). AT-III could not be measured in the presence of HBOC (due to color interference). However, by 48 to 72 h (when HBOC was cleared), measurements were at baseline in all three groups. Thrombin time (baseline, 18.1 ± 1.9 s) remained unchanged throughout the experimental period and was similar in all three groups.

Albumin and total protein levels were similar in NON animals during the course of the experiment. Albumin decreased in the prehospital phase in fluid resuscitated animals due to hemodilution. AST peaked at 24 h in all three groups, but with remarkable amplification (>10-fold from baseline) in HBOC compared with HEX and NON animals (each about 6-fold; group and time effects, $P < 0.01$; Fig. 7).

EM

On EM of the lungs, there was a trend toward a higher rate of alveolar edema in NON animals ($P > 0.05$; Fig. 8). Small amounts of fibrin deposition were observed in all three groups. However, no platelet aggregates or microthrombi were found in any of the animals.
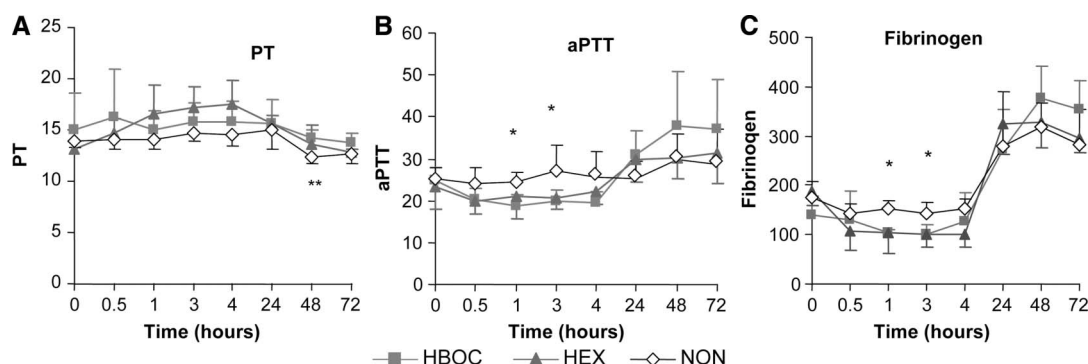
Fig. 6. **Coagulation parameters for 40% controlled hemorrhaged swine**. (A) PT, (B) aPTT, (C) fibrinogen for HBOC- (—■—), HEX- (—▲—), and NON-resuscitated pigs (—◇—). Values are presented as mean ± SD, *$P < 0.01$ between NON and treated animals; **$P < 0.02$ between baseline and 48 h for PT.

DISCUSSION

Coagulopathy is a common but as yet poorly managed complication of HS that is characterized by events that are complex and multifactorial in etiology. Excessive fibrinolysis, consumption of platelets and coagulation factors, hemodilution, hypothermia, and acidosis occur after HS and contribute to an imbalance of coagulation factors as well as dysregulation of the coagulation cascade (3, 5, 22, 23). Ultimately, coagulopathy caused increased morbidity and mortality (11, 13, 24). Survival of the trauma patient relies, in part, on preventing escalation of coagulopathy to events such as DIC and hypofibrinogenemia (25, 26). Although animal studies have limitations such as species-specific and anesthesia responses in controlled experiments, animal data are important (26).

In the controlled hemorrhage model described here, all animals experienced the same loss in cell numbers and fluid volume due to hemorrhage before the initiation of resuscitative treatments. In classical fluid shift dynamics after HS, extravasation from tissue to circulation and increased extracellular protein is a common response to sustain isovolemia (27, 28). However, we found that in the NON group, hemoconcentration occurred early during the prehospital phase as evidenced by increased hematocrit, possibly a consequence of intravascular to extravascular fluid movement. Because pigs were not splenectomized, theoretically, this could be due to self-transfusion through a contractile spleen (28, 29). In the hospital phase, changes in hematocrit for all the groups correlated with saline and blood transfusion requirements. These findings may affect therapeutic strategies for HS because restoration of blood cellular mass is reported to be as important as the establishment of fluid volume (30).

The lack of BT changes in both treatment groups reflects absence of significant hypercoagulability effects by either fluid. However, decreased BT during the prehospital phase in NON animals may be due to higher hematocrit (31).

Despite posthemorrhage hemoconcentration in NON animals, platelet concentration was reduced in this group. Platelet sequestration may partially explain this phenomenon. This possibility is supported by the fact that there was only minimal tissue trauma (abdominal muscle crush) in this controlled hemorrhage model and EM findings failed to show microthrombi or adherence of platelet to endothelium. Such observations indicate a lack of platelet activation or recruitment of new platelets.
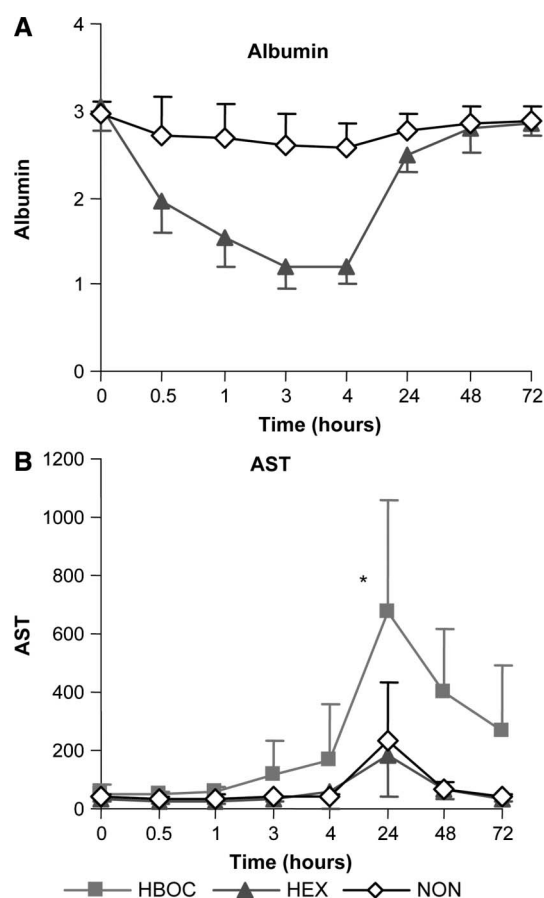

Fig. 7. **Chemistry parameters for 40% controlled hemorrhaged swine**. (A) Albumin in HEX- and NON-treated pigs. (B) Hepatic enzyme AST for HBOC- (—■—), HEX- (—▲—), and NON-resuscitated pigs (—◇—). Values are presented as mean ± SD, *$P < 0.01$ between HBOC and HEX or NON pigs.

The similar decline in total number of platelets in all three groups during the prehospital phase may reflect mechanisms independent of fluid resuscitation but processes initiated by loss of blood volume and MAP. Interestingly, circulating platelets are known to disappear in hibernating animals as blood flow slows with the onset of torpor; platelets subsequently reappear in the arousal phase when blood flow increases (32). Although circulating platelets have been noted to increase with restoration of MAP in canine and swine models of HS (33, 34),
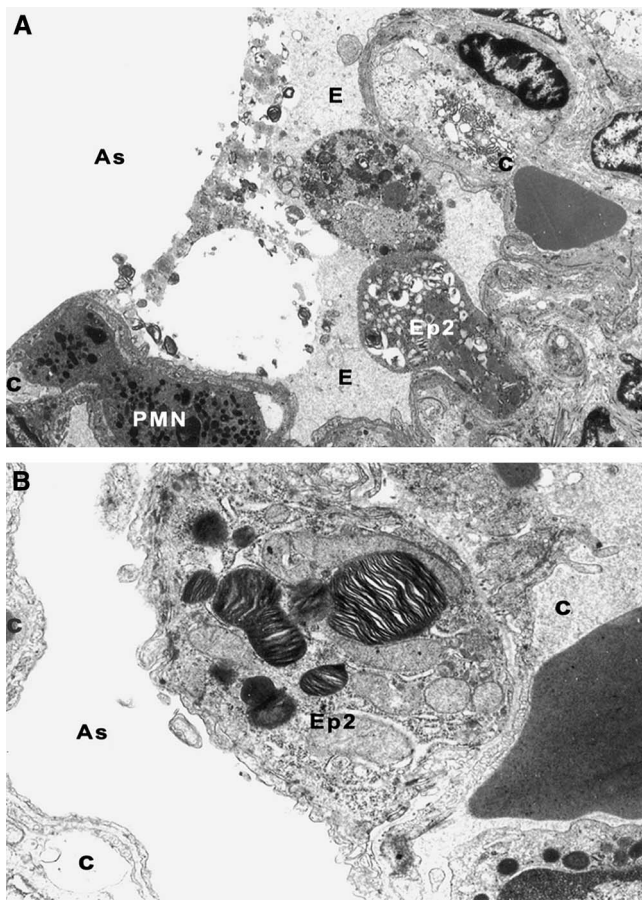
Fig. 8. **EM**. (A) Electron micrograph of the lung from a NON-resuscitated pig shows alveolar edema (E) (×2900). (B) Electron micrograph of the lung from a HBOC-resuscitated pig shows normal alveoli edema (E; ×7250). As, alveolar space; C, capillary; Ep2, type 2 epithelial cell; PMN, polymorphonuclear leukocyte.

the observed increase during the hospital phase in HEX animals was probably largely due to blood transfusions. By comparison, in HBOC and NON groups, where Hb levels remained above the target of 7 g/dL and animals were not transfused, platelet concentrations did not increase.

In addition to platelet dynamics, functional assays of coagulation were used to examine the effects of hemorrhage and resuscitation on hemostasis. In resuscitated animals, the observed reduction in platelet concentration was not sufficient to explain the prolonged PFA-CT in the prehospital phase. PFA-CT (*in vitro* bleeding), which addresses platelet function independent of *in vivo* parameters (such as MAP), was likely prolonged by hemodilution of vWF and low hematocrit, both consequences of fluid resuscitation. The elevated PFA-CT supports findings relative to the coagulopathic effects of HEX (27, 38) suggesting less effect of HBOC-201 than HEX on platelets and/or vWF activity. In the hospital phase, the PFA-CT remained elevated for HBOC, even after physiologic stability, suggesting a more persistent although relatively mild effect of HBOC, probably because of differences in blood transfusions at 4 h. In HEX and HBOC animals, the return of PFA-CT values to baseline coincided with increased numbers of circulating platelets and possibly circulating levels of vWF (35). PFA-CT was also increased in NON animals during the

hospital phase, likely because of saline infusion (seven of seven NON animals received saline at 240 min). In contrast with previous findings with *o*-raffinose polymerized hemoglobin (Hemolink) (19), there was no evidence of platelet stimulation with HBOC-201. Although experimental conditions were not identical, the dose of HBOC infused in the present study was higher (2.3 g/kg) than in the Hemolink study (0.8 g/kg) (16).

TEG-CI, a general index for coagulation, did not change in NON-treated animals during the prehospital phase but decreased in resuscitated groups due to hemodilution. In the hospital phase, this pattern resolved at 24 h in HEX animals likely due to blood transfusions. In contrast, HBOC animals remained mildly hypocoagulopathic at 24 h and did not normalize until 48 h after blood transfusions. The absence of excess fibrinolysis (unchanged TEG-Ly) suggested the absence of DIC. In the prehospital phase, there were no dramatic differences in TEG-R, TEG-K, or TEG-α in NON animals. These results suggest that there was no hypercoagulation or platelet activation due solely to HS. Similar results were also observed in HEX animals. In HBOC animals, these parameters increased 3 h posthemorrhage and attained maximum values at 24 h. This effect paralleled PFA-CT observations and may be related to residual plasma HBOC that was cleared 3 days posthemorrhage.

PT remained unchanged throughout the 4-h prehospital phase in all groups, confirming TEG data, and suggesting that significant hepatic malfunction did not occur. However, aPTT in treated, but not NON animals, was mildly decreased during the prehospital phase, suggesting that fluid resuscitation induced some compensatory shift toward the intrinsic coagulation pathway. This difference was observed even in the presence of hemodilution. In the hospital phase, HBOC, but not HEX animals, had mildly prolonged aPTT. Disturbance in the TEG pattern (elevated TEG-R and reduced TEG-CI at 24 h) indicated some impairment of clot formation. Moreover, this combination of normal PT, prolonged aPTT, and elevated TEG-R in the posthospital phase suggests possible elevated circulating heparin. Despite elevated AST in HBOC animals, however, normal PT at 24 h suggests normal liver synthetic function. Nevertheless, the pathophysiology of HBOC-induced elevation in liver function tests has not been well studied and it is possible that observed LFT abnormalities could be more clinically significant in patients with pre-existing liver disease. Further research is needed in this area.

The initial drop in fibrinogen in treated animals during the prehospital phase reflected hemodilution, as well as possible consumption during HS. The increased fibrinogen (~3-fold) seen in the hospital phase was probably mostly because of *de novo* synthesis of fibrinogen (acute phase reactant). As this increase was seen also in HBOC pigs, which did not receive transfusions until 24 h, a significant contribution by blood transfusions is unlikely. AT-III levels were also affected by dilution as seen in the HEX group. The effect of dilution on regulation of the intrinsic pathway awaits further study. Lactate increased in all groups, suggesting microvascular hypoperfusion and capillary bed constriction soon after hemorrhage, as reported by others (36). Our results supplement other reports on the effects of HBOC-201 on physiology and survival in HS in

swine (8, 9, 37). However, the results of these studies differed from a recently published article with human polymerized hemoglobin (Polyheme; Northfield Laboratories, Evanston, IL) (38). As this study summarized only physiology findings, evaluated a different HBOC, and used different hemorrhage and animal models, direct comparisons between the two HBOCs cannot be made.

In conclusion, in this comprehensive study of coagulation in a controlled HS model, no significant hypo- or hypercoagulation was observed in the prehospital phase. However, HBOC-201 resuscitation was associated with mild dilutional hypocoagulation in the hospital phase, at least partially related to lower transfusion requirements. Within a few days of hospitalization, administration of blood components may be required in HBOC-resuscitated patients with persistent hemorrhage. As restoration of hemodynamics and tissue oxygenation occurred with lower fluid requirements, and without development of clinically significant coagulopathy or thrombopathy, HBOC-201 was at least as efficacious as HEX, as a prehospital resuscitative fluid for hemorrhagic shock.

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REFERENCES

1. Champion HR, Bellamy RF, Roberts CP, Leppaniemi A: A profile of combat injury. *J Trauma* 54:S13–S19, 2003.
2. Hoyt DB: Fluid resuscitation: the target from an analysis of trauma systems and patient survival. *J Trauma* 54 (5 Suppl):S31–S35, 2003.
3. McNamara JJ, Burran EL, Stremple JF, Molot MD: Coagulopathy after major combat injury: occurrence, management, and pathophysiology. *Ann Surg* 176:243–246, 1972.
4. Murray CJ, Lopez AD: Alternative projections of mortality and disability by cause 1990–2020: Global Burden of Disease Study. *Lancet* 349:1498–1504, 1997.
5. Heckbert SR, Vedder NB, Hoffman W, Winn RK, Hudson LD, Jurkovich GJ, Copass MK, Harlan JM, Rice CL, Maier RV: Outcome after hemorrhagic shock in trauma patients. *J Trauma* 45:545–549, 1998.
6. Page TC, Light WR, McKay CB, Hellums JD: O_2 transport by erythrocyte/Hb solution mixtures in an *in vitro* capillary as a model of Hb-based blood substitute performance. *Microvasc Res* 55:54–64, 1998.
7. Levy JH, Goodnough LT, Greilich PE, Parr GV, Stewart RW, Gratz I, Wahr J, Williams J, Comunale ME, Doblar D, Silvay G, Cohen M, Jahr JS, Vlahakes GJ: Polymerized bovine hemoglobin solution as a replacement for allogeneic red blood cell transfusion after cardiac surgery: results of a randomized, double-blind trial. *J Thorac Cardiovasc Surg* 124:35–42, 2002.
8. Katz LM, Manning JE, McCurdy S, Pearce LB, Gawryl MS, Wang Y, Brown C: HBOC-201 improves survival in a swine model of hemorrhagic shock and liver injury. *Resuscitation* 54:77–87, 2002.
9. Sampson JB, Davis MR, Mueller DL, Kashyap VS, Jenkins DH, Kerby JD: A comparison of the hemoglobin-based oxygen carrier HBOC-201 to other low-volume resuscitation fluids in a model of controlled hemorrhagic shock. *J Trauma* 55:747–754, 2003.
10. Philbin N, Rice J, Gurney J, McGwin G, Arnaud F, Dong F, Johnson T, Flournoy WS, Ahlers S, Pearce LB, McCarron R, Freilich D: A hemoglobin based oxygen carrier, bovine polymerized hemoglobin (HBOC-201) versus hetastarch (HEX) in a moderate severity hemorrhagic shock swine model with delayed evacuation. *Resuscitation.* 2005 (in press).
11. Brohi K, Singh J, Heron M, Coats T: Acute traumatic coagulopathy. *J Trauma* 54:1127–1130, 2003.
12. Lapointe LA, Von Rueden KT: Coagulopathies in trauma patients. *AACN Clin Issues* 13:192–203, 2002.
13. Kearney TJ, Bentt L, Grode M, Lee S, Hiatt JR, Shabot MM: Coagulopathy and catecholamines in severe head injury. *J Trauma* 32:608–611, 1992.
14. Jamnicki M, Bombeli T, Seifert B, Zollinger A, Camenzind V, Pasch T, Spahn DR: Low- and medium-molecular-weight hydroxyethyl starches: comparison of their effect on blood coagulation. *Anesthesiology* 93:1231–1237, 2000.
15. Tabuchi N, de Haan J, Gallandat Huet RC, Boonstra PW, van Oeveren W: Gelatin use impairs platelet adhesion during cardiac surgery. *Thromb Haemost* 74:1447–1451, 1995.
16. Lee DH, Bardossy L, Peterson N, Blajchman MA: *o*-Raffinose cross-linked hemoglobin improves the hemostatic defect associated with anemia and thrombocytopenia in rabbits. *Blood* 96:3630–3636, 2000.
17. Pearce LB, Gawryl MS: Overview of preclinical and clinical efficacy of Biopure's HBOCs. In Chang TMS (ed): *Blood Substitutes: Principles, Methods, Products, and Clinical Trials*. Basel, Switzerland: Karger Landes Systems, 1998, pp 88–110.
18. Jahr JS, Lurie F, Driessen B, Davis JA, Gosselin R, Gunther RA: The HemoCue, a point of care B-hemoglobin photometer, measures hemoglobin concentrations accurately when mixed *in vitro* with canine plasma and 3 hemoglobin-based oxygen carriers (HBOC). *Can J Anaesth* 49:243–248, 2002.
19. Kaufmann CR, Dwyer KM, Crews JD, Dols SJ, Trask AL: Usefulness of thrombelastography in assessment of trauma patient coagulation. *J Trauma* 42:716–720, 1997.
20. Favaloro EJ: Utility of the PFA-100 for assessing bleeding disorders and monitoring therapy: a review of analytical variables, benefits and limitations. *Haemophilia* 7:170–179, 2001.
21. Asher LV, Binn LN, Mensing TL, Marchwicki RH, Vassell RA, Young GD: Pathogenesis of hepatitis A in orally inoculated owl monkeys (*Aotus trivirgatus*). *J Med Virol* 47:260–268, 1995.
22. De Waele JJ, Vermassen FE: Coagulopathy, hypothermia and acidosis in trauma patients: the rationale for damage control surgery. *Acta Chir Belg* 102:313–316, 2002.
23. Rotondo F, Keilly PM: Bleeding and coagulation complications. In Matox K, Feliciciano D, Moore E (ed): *Trauma*. New York: McGraw-Hill, 2001, pp 1267–1285.
24. MacLeod JB, Lynn M, McKenney MG, Cohn SM, Murtha M: Early coagulopathy predicts mortality in trauma. *J Trauma* 55:39–44, 2003.
25. Hulka F, Mullins RJ, Frank EH: Blunt brain injury activates the coagulation process. *Arch Surg* 131:923–927, 1996.
26. Ledgerwood AM, Lucas CE: A review of studies on the effects of hemorrhagic shock and resuscitation on the coagulation profile. *J Trauma* 54:S68–S74, 2003.
27. Rothe CF, Drees JA: Vascular capacitance and fluid shifts in dogs during prolonged hemorrhagic hypotension. *Circ Res* 38:347–356, 1976.
28. Hannon JP, Bossone CA, Rodkey WG: Splenic red cell sequestration and blood volume measurements in conscious pigs. *Am J Physiol* 248:R293–R301, 1985.
29. Stewart IB, McKenzie DC: The human spleen during physiological stress. *Sports Med* 32:361–369, 2002.
30. Simmons RL, Collins JA, Heisterkamp CA, Mills DE, Andren R, Phillips LL: Coagulation disorders in combat casualties. I. Acute changes after wounding. II. Effects of massive transfusion post-resuscitative changes. *Ann Surg* 169:455–482, 1969.
31. Feffer SE: Hematocrit and bleeding time: an update. *South Med J* 87:299–301, 1994.
32. Boyer BB, Barnes BM: Molecular and metabolic aspects of mammalian hibernation. *Bioscience* 49:713–724, 1999.
33. Martin BA, Dahlby R, Nicholls I, Hogg JC: Platelet sequestration in lung with hemorrhagic shock and reinfusion in dogs. *J Appl Physiol* 50:1306–1312, 1981.
34. Blomqvist S, Thorne J, Elmer O: Different effects of bleeding and soft-tissue trauma on pulmonary platelet trapping in pigs. *J Trauma* 29:866–872, 1989.
35. Strauss RG, Stump DC, Henriksen RA: Hydroxyethyl starch accentuates von Willebrand's disease. *Transfusion* 25:235–237, 1985.
36. Alam HB, Uy GB, Miller D, Koustova E, Hancock T, Inocencio R, Anderson D, Llorente O, Rhee P: Comparative analysis of hemostatic agents in a swine model of lethal groin injury. *J Trauma* 54:1077–1082, 2003.
37. Gurney J, Philbin N, Rice J, Arnaud F, Dong F, Wulster-Radcliffe M, Pearce B, Kaplan L, McCarron R, Freilich D: A hemoglobin based oxygen carrier, bovine polymerized hemoglobin (HBOC-201) versus hetastarch (HEX) in an un-controlled liver injury hemorrhagic shock swine. *J Trauma* 57:726–738, 2004.
38. Handrigan MT, Bentley TB, Oliver JD, Tabaku LS, Burge JR, Atkins JL: Choice of fluid influences outcome in prolonged hypotensive resuscitation after hemorrhage in awake rats. *Shock* 23:337–343, 2005.